Materials and Methods

Cell Culture

Human aortic SMC (human SMC) were isolated, characterized, and immortalized as previously described. Primary human aortic SMC were obtained from Clonetics Corporation (Palo Alto CA). Cells were routinely cultured in growth media (GM, DMEM containing 15% FBS, 1.4 mmol/L phosphate, 100 U/mL of penicillin and 100 mg/mL of streptomycin). For elevated phosphate conditions, GM was supplemented with NaH$_2$PO$_4$/Na$_2$HPO$_4$ to a final concentration of 2.6 mmol/L (calcification media, CM).

Preparation of Stable SMC Expressing Pit-1 Small Interfering RNA (siRNA)

The pSUPER RNA interference system (Oligoengine, Seattle, WA) was used to generate SMC stably expressing Pit-1 siRNA. Briefly, oligonucleotides for human Pit-1 (5’GCCTGAAGTATCTCTCCTC3’) or Pit-1 control (5’TCCAGAGGTATGCCACATA3’) were obtained from MWG Biotech Inc (High Point, NC). The annealed double-stranded oligonucleotides were inserted into the pSUPER retrovirus vector, and recombinant constructs were transfected into Phoenix packaging cells to generate retrovirus. The infection of SMC was performed using polybrene by exposure to retrovirus for 1 h every 24 h for three consecutive days. The infected cells were selected with 3 µg/ml puromycin for 72 h. SMC containing Pit-1 siRNA are hereafter referred to as SMC-iRNA, whereas the SMC containing the Pit-1 control construct are referred to as SMC-CT.
Reverse Transcription PCR and Preparation of cDNA Probes

Total RNA was isolated from cultured SMC using RNeasy kit from Qiagen (Chatsworth, CA). Reverse transcription was performed using First-Strand cDNA Synthesis kit (Amersham Biosciences UK, England). The primers used for PCR amplification were: 1) Human Pit-1 forward 5'-TACCATCCTCATCTCGGTGG-3' and reverse 5'-TGACGGCTTGACTGAAGCTG-3', the amplified fragment corresponds to base pairs 1060 - 1469 of human Pit-1 (L20859). 2) Human Pit-2 forward 5'-TGGATGGTCATTTTGGGTTT-3' and reverse primer 5'-GCACACCTTTGTAAGCGATT-3’, the amplified fragment corresponds to base pairs 265-649 of human Pit-2 (L20852). 3) Mouse Pit-1 forward 5'-TACCATCCTCATCTCGGTGG-3' and reverse 5'-TGACAGTTTGACTGAACTGA -3’. The PCR product is 406 base pairs and corresponds to position 1111-1517 of mouse Pit-1 (M73696). 4) Human sodium hydrogen antiporter (NHA) forward 5'-GATCCTTCTGGCCTGCCTCA-3' and reverse primer 5'-TCAGGATGGTCGCCAGGT-3’, which corresponds to base pairs 377-624 of the human NHA (M81768). The amplified fragments were obtained by RT-PCR from the mRNA of SMC, and then subcloned into the TA cloning vector (Invitrogen, Carlsbad, CA) previously described. The identity of the cDNA inserts as Pit-1, Pit-2 or NHA was confirmed by DNA sequence analysis.

Cloning and Expression of Mouse Pit-1

Full-length mouse Pit-1 cDNA was cloned from mouse aortic SMC by RT-PCR, using the forward primer 5’ GGATCCATGGAATCTACTGTGGCAACG -3’ and the reverse
primer 5’-CTCGAGTCACACTGGCAGGATGATGT-3’. The PCR product was cloned into the EcoR I site of the retroviral expression vector pLXIN (BD Biosciences, Palo Alto, CA). Recombinant pLXIN containing mouse Pit-1 cDNA or empty vector were transfected into the Phoenix packaging cell line by calcium-phosphate precipitation to generate retrovirus. Retroviral infection of SMC-iRNA was performed as described above. The transduced cells containing mouse Pit-1 or empty vector were referred to as SMC-mPit1 or SMC-LXIN respectively. Pooled populations of cells were used for experiments.

**Quantitative Real-Time PCR**

Total RNA was isolated from human SMC using the RNeasy kit from Qiagen (Chatsworth, CA). Reverse transcription was performed using Omniscript Reverse Transcriptase from Qiagen. Levels of human Pit-1, Pit-2, Cbfa-1 and OPN mRNAs were determined by quantitative real-time PCR performed with TaqMan PCR reagents kits in the ABI Prim 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers and probes were designed using Primer-Express software V2.0 (Applied Biosystems). The primers were synthesized by Qiagen and the Taq probes were obtained from the Applied Biosystems. All of the Taq probes have a FAM (fluorochrome reporter) tag at the 5’-end and an MGB quencher at the 3’-end and the sequences are: 1) Human Pit-1 forward 5’-GGAGGGTGTAAGTGGATCTCTGGGT-3’ and reverse 5’-ATCTGCTTATGGAGGATGAATG -3’, the probe 5’-CTGATAAAAATTGTGATGCTTTGG-3’. 2) Human Pit-2 forward 5’-forward-
TACAACGAGACGGTGGAGACTCT-3' and reverse 5'-
AAGCAATCAGCTGCCACACA-3', the probe 5'-CCATGGTTGGTCCG-3'.
3) Human Cbfa-1 forward 5'- CCCGTGGCCTTCAAGGT-3' and reverse 5'-
TGACAGTAACCACAGTCCCATCTG-3', the probe 5'-AGCCCTCGGAGAGGT-3'.
4) Human OPN forward 5'- TGTCCTCTGAAGAAACCAATGACTT -3' and reverse 5'-
TCATGGCTTTTCGTTGGACTTACT -3', the probe 5'-AAACAAGAGACCCTTCC -3'.
All PCR reactions were performed in triplicate. Quantification of gene expression was calculated
by the standard curve method according to the manufacturer’s protocol and normalized to 18S rRNA.

Northern Blot Analysis
Total RNA was isolated from SMC with Trizol (Invitrogen Inc., Carlsbad, CA). Total RNA was electrophoresed, transferred to a Zeta-probe blotting membrane (Bio-Rad Laboratories, Hercules, CA), and hybridized with \(^{32}\)P-labeled cDNA probes for human Pit-1, Pit-2 or NHA as previously described.\(^1\) Relative mRNA levels were quantified by densitometric scanning and normalized to 18S rRNA. The sequence of 18S rRNA probe was described previously.\(^2\)

Phosphate Uptake Assays
Phosphate uptake assays were performed as previously described.\(^1\) In brief, SMC were incubated in Earle’s buffered salt solution (EBSS) containing various concentrations of phosphate (including \(H_3^{32}\)PO\(_4\) obtained from PerkinElmer Life Science, Inc. Boston, MA). Sodium-dependent phosphate uptake was determined by subtracting uptake in the
presence of EBSS containing choline chloride from uptake in EBSS containing sodium chloride. Uptake values were normalized to cellular protein content.

**Calcium Quantitation**

SMC calcification was induced by the treatment with calcification media (CM, 2.6 mmol/L phosphate). Calcium content of the cultures was determined using the \(O\)-cresolphthalein complexone method exactly as previously described\(^1\) and normalized to protein content.

**Western Blot Analysis**

Cell lysates were prepared as described previously.\(^2\) Equal amounts of protein were loaded and separated by 8% SDS-PAGE followed by transfer to PVDF membrane. The membranes were then blotted using anti-Pit-1 or anti-Annexin V antibodies (R&D System, Minneapolis, MN). Pit-1 antibody was obtained by immunizing rabbits with the Pit-1 peptide, QAVVEERTVSFKLGDLEEAPERERLPSVDLKEETSIDSTV, followed by peptide affinity purification of serum using Sulfo-Link kit from Pierce Biotechnology, Inc. (Rockford, IL). Specific proteins were detected with enhanced chemiluminescence reagents (Perkin Elmer Life Science, Inc. Boston, MA).

**Membrane-bound Vesicle Isolation**

Membrane-bound, extracellular vesicles were prepared by two different methods as described previously.\(^3,4\) For extracellular matrix-derived vesicles, after 2 day incubation with normal growth media (GM, containing 1.4 mmol/L phosphate) or calcification
media (CM, containing 2.6 mmol/L phosphate), SMC monolayers were washed and then incubated with 500 U/ml of collagenase for 3 hours at 37°C. The digest was centrifuged for 20 mins at 15,000g to pellet cell debris. The supernatant was centrifuged for 60 min at 100,000g. The resulting pellet containing extracellular matrix-derived vesicles was resuspended and used for subsequent experiments. Alternatively, SMC were incubated overnight with either serum-free media (SFM, serum-free GM supplemented with 0.5% BSA) or elevated calcium and phosphate containing media (CPM, serum-free media with 2.7 mmol/L calcium and 2.0 mmol/L phosphate). The culture medium was collected and centrifuged for 20 min at 2500 rpm to remove the debris, the supernatant (containing shed membrane-bound vesicles) was then spun for 30 min at 100,000g.

**Apoptosis Assay**

The Cell Death Detection ELISA kit (Roche Diagnostics Co, Indianapolis, IN) was used according to the manufacturer’s direction to quantitatively determine cytoplasmic histone-associated DNA oligonucleosome fragments associated with apoptotic cell death.\(^5\)

**Statistical Analysis**

Results are expressed as mean ± SD. Significance between groups was determined by ANOVA, \(p\)-values less than 0.05 were considered significant.
References


